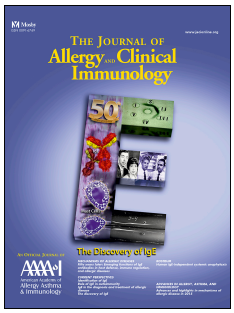
Mer-mediated eosinophil efferocytosis regulates resolution of allergic airway inflammation

Accepted Manuscript



Mer-mediated eosinophil efferocytosis regulates resolution of allergic airway inflammation

Jennifer M. Felton, PhD, Christopher D. Lucas, MD PhD, David A. Dorward, MD PhD, Rodger Duffin, PhD, Tiina Kipari, PhD, Sonja Vermeren, PhD, Calum T. Robb, PhD, Kenneth G. MacLeod, Bryan Serrels, PhD, Jürgen Schwarze, MD PhD, Christopher Haslett, MD, Ian Dransfield, PhD, Adriano G. Rossi, PhD DSc

PII: S0091-6749(18)30215-X

DOI: 10.1016/j.jaci.2018.01.029

Reference: YMAI 13284

To appear in: *Journal of Allergy and Clinical Immunology*

Received Date: 16 October 2017

Revised Date: 7 December 2017

Accepted Date: 5 January 2018

Please cite this article as: Felton JM, Lucas CD, Dorward DA, Duffin R, Kipari T, Vermeren S, Robb CT, MacLeod KG, Serrels B, Schwarze J, Haslett C, Dransfield I, Rossi AG, Mer-mediated eosinophil efferocytosis regulates resolution of allergic airway inflammation, *Journal of Allergy and Clinical Immunology* (2018), doi: 10.1016/j.jaci.2018.01.029.

Mer-mediated eosinophil efferocytosis regulates resolution of allergic airway inflammation

Jennifer M. Felton*[1] PhD, Christopher D. Lucas*[1] MD PhD, David A. Dorward[1] MD PhD, Rodger Duffin[1] PhD, Tiina Kipari[1] PhD, Sonja Vermeren[1] PhD, Calum T. Robb[1] PhD, Kenneth G. MacLeod[2], Bryan Serrels[2] PhD, Jürgen Schwarze[1] MD PhD, Christopher Haslett[1] MD, Ian Dransfield[1] PhD, Adriano G. Rossi[1] PhD DSc.

[1] MRC Centre for Inflammation Research, The Queen's Medical Research Institute, University of Edinburgh, 47 Little France Crescent, Edinburgh, Scotland, UK, EH16 4TJ.

[2] MRC Institute of Genetics and Molecular Medicine, University of Edinburgh, Western General Hospital Campus, Crewe Road, Edinburgh, EH4 2XU.

* Joint first authors

Corresponding author: CD Lucas, Room W2.01, MRC Centre for Inflammation Research, Queen's Medical Research Institute, University of Edinburgh, Scotland, UK, EH16 4TJ. Tel: +44 131 2426662, email: christopher.lucas@ed.ac.uk

Funding: The authors acknowledge funding from the Wellcome Trust 206566/Z/17/Z (CDL), WT096497 (DAD), the UK Medical Research Council (MR/K013386/1: AGR, CH, RD, CTR, TK and JMF) and a Wellcome Trust-University of Edinburgh Institutional Strategic Support Fund (1S2-101/02: CDL).

Abstract

Background: Eosinophils play a central role in the propagation of allergic diseases including asthma. Both recruitment and retention of eosinophils regulate pulmonary eosinophilia but the question of whether alterations in apoptotic cell clearance by phagocytes directly contributes to resolution of allergic airway inflammation remains unexplored.

Objectives: In this study we investigated the role of the receptor tyrosine kinase Mer in mediating apoptotic eosinophil clearance and allergic airway inflammation resolution *in vivo* in order to establish whether apoptotic cell clearance directly impacts upon the resolution of allergic airway inflammation.

Methods: Alveolar and bone-marrow macrophages were used to study Mer-mediated phagocytosis of apoptotic eosinophils. Allergic airway inflammation resolution was modelled in mice using ovalbumin. To determine apoptotic cell clearance *in vivo*, fluorescently labeled apoptotic cells were administered intratracheally or eosinophil apoptosis was driven by administration of dexamethasone.

Results: Inhibition or absence of Mer impaired phagocytosis of apoptotic human and mouse eosinophils by macrophages. Mer-deficient mice displayed delayed resolution of ovalbumin-induced allergic airway inflammation together with increased airway responsiveness to aerosolized methacholine, elevated bronchoalveolar lavage fluid protein levels, altered cytokine production and an excess of uncleared dying eosinophils after dexamethasone treatment. Alveolar macrophage phagocytosis was significantly Mer-dependent, with the absence of Mer attenuating apoptotic cell clearance *in vivo* to enhance inflammation in response to apoptotic cells.

Conclusions: We demonstrate that Mer-mediated apoptotic cell clearance by phagocytes contributes to resolution of allergic airway inflammation, suggesting that augmenting apoptotic cell clearance is a potential therapeutic strategy for treating allergic airway inflammation.

Key Messages

- Mer drives clearance of apoptotic eosinophils, key cells in allergic airway inflammation.
- Absence of Mer leads to delayed resolution of inflammation and increased airway resistance in allergic airway inflammation.
- Augmenting apoptotic cell clearance is therefore a potential therapeutic strategy for treating allergic inflammation.

Capsule Summary

Defective apoptotic cell clearance is observed in allergic diseases including asthma, but whether this directly contributes to pathophysiology is unclear. Using Mer deficient mice, we demonstrate that impaired apoptotic cell clearance exacerbates allergic airway inflammation.

Key Words

Eosinophil, Apoptosis, Phagocytosis, MerTK, Inflammation Resolution, Allergic airway Inflammation, Airway Resistance.

Abbreviations

AC:	Apoptotic cells
AMs:	Alveolar macrophages
BALF:	Bronchoalveolar lavage fluid
BMDMs:	Bone marrow-derived macrophages
bmEos:	Bone marrow-derived eosinophils
DMEM:	Dulbecco's modified Eagle Medium
FCS:	Fetal calf serum
FLT3-L:	FMS-like tyrosine kinase 3 ligand
H&E:	Hematoxylin and eosin
IMDM:	Iscove’s modified Dulbecco’s medium
i.p.	Intraperitoneal

i.t.	Intratracheal
LPS:	Lipopolysaccharide
Mer^{KD}:	Mer-deficient/kinase dead
OVA:	Ovalbumin
PAS:	Periodic acid-Schiff
PBS:	Phosphate-buffered saline
R_i:	Resolution interval
Pros1:	Protein S
RPMI:	Roswell Park Memorial Institute 1640 medium
SCF:	Stem cell factor
TAM:	Tyro-3/Axl/Mer
TLR:	Toll-like receptor
WT:	Wild type

Introduction

Eosinophils play a major role in the propagation of allergic airway diseases such as asthma[1,2]. During inflammation, eosinophils are recruited from the bone marrow and migrate to inflamed tissue where they can release a range of cytotoxic eosinophil-derived products that promote inflammation, tissue remodelling, airway hyperresponsiveness and organ dysfunction[3].

Tissue presence of eosinophils is determined by both recruitment and retention within inflamed sites. Eosinophil elimination from the lung can be regulated by transepithelial migration and mucociliary clearance, or by apoptosis and subsequent phagocytosis by macrophages, dendritic cells and airway epithelial cells, a process termed efferocytosis[4]. The relative role and importance of eosinophil apoptosis and efferocytosis in the resolution of allergic airway inflammation in humans remains controversial[5] but several lines of evidence suggest that these pathways have relevance to allergic disease states. Prolonged eosinophil longevity (with reduced apoptosis) associates with increasing asthma severity in humans[6], while macrophages from individuals with severe or poorly controlled asthma have defective efferocytosis[7,8]. In addition, we have recently shown that driving eosinophil apoptosis with the flavone wogonin attenuates allergic lung inflammation in mice *in vivo*[9], suggesting that modulation of eosinophil apoptosis is a *bona fide* target for treating allergic diseases. The question of whether alterations in apoptotic cell clearance by phagocytes directly contributes to resolution of allergic airway inflammation remains to be addressed.

Although the molecular mechanisms driving changes in eosinophil lifespan and clearance *in vivo* remain poorly defined, it is known that glucocorticoids, the main treatment for asthma and other allergic diseases, induce eosinophil apoptosis and upregulate macrophage phagocytosis of apoptotic cells *in vitro*[10,11]. Glucocorticoid-augmented efferocytosis is dependent upon Mer[11], a member of the Tyro-3/Axl/Mer (TAM) receptor tyrosine kinase family[12]. There are two well-defined ligands for Mer, Protein S (Pros1) and Gas6, which can bridge to phosphatidylserine exposed on apoptotic cells. The importance of TAM receptors and their ligands in efferocytosis has been demonstrated using Mer-deficient (Mer^{KD}) and triple TAM-deficient mice. These mice are characterised by impaired efferocytosis in lymphoid tissues, diminished apoptotic germ cell removal by Sertoli cells in the testis and defective pruning of the photoreceptors in the retina by the retinal pigment epithelial cells[13–

[15]. Previous studies have investigated the role of Mer in neutrophil-dominant lung injury models (induced by lipopolysaccharide (LPS) and bleomycin)[16], while Axl downregulation has been demonstrated in moderate-severe human asthma[17]. However, the potential role of Mer in regulating eosinophil clearance and resolution of allergic airway inflammation remains unexplored.

In the present study, we investigated Mer-mediated eosinophil efferocytosis and its role in allergic airway inflammation resolution *in vivo,* to establish whether apoptotic cell clearance directly impacts upon the resolution of allergic airway inflammation. Absence or inhibition of Mer impaired phagocytosis of apoptotic human and mouse eosinophils by macrophages, while Mer-deficient mice had delayed resolution of ovalbumin (OVA)-induced allergic airway inflammation.

Methods

Eosinophil isolation

Human granulocytes were isolated from blood of healthy volunteers as described[19] (Lothian Research Ethics Committee (#08/S1103/38; #15-HV-013)). Eosinophils were subsequently isolated by anti-CD16^{+ve} microbeads (Miltenyi Biotec, #130-045-701) following manufacturer's instructions with purity >95% as assessed by cellular morphology of Diff-Quik stained cytocentrifuge preparations. Cells were cultured in Iscove's modified Dulbecco's medium (IMDM; Gibco) with 10% autologous serum (37°C/5% CO_2).

Mouse bone marrow-derived eosinophils (bmEos) were generated from unselected bone marrow progenitor cells using an extended 14 day version of a described protocol[20]. Briefly, bone marrow cells were cultured in Roswell Park Memorial Institute 1640 medium (RPMI 1640; Gibco), supplemented with 20% FCS (fetal calf serum), 100 IU/mL Penicillin/Streptomycin, 2mM L-glutamine, 25mM HEPES (Sigma), 1x nonessential amino acids and 1mM sodium pyruvate (both Gibco), 50μM 2-mercaptoethanol, stem cell factor (SCF; 100ng/mL, PeproTech) and FLT3-ligand (FLT3-L; 100ng/mL, PeproTech) for the first 4 days before switching to media containing IL-5 (10mg/mL, PeproTech) for the remainder of the culture period. After 14 days, cells were >95% eosinophils as assessed by cellular morphology and expression of Siglec-F by flow cytometry.

Macrophage isolation

Mouse bone marrow-derived macrophages (BMDMs) were generated as described[21]. Tibias and femurs were flushed with Dulbecco's modified Eagle Medium (DMEM; Gibco) and red blood cells lysed with ACK lysis buffer (Gibco) prior to passing through a 40μm cell strainer. Cells were plated onto 15cm cell culture dishes (Corning) in DMEM with 20% FCS, 100 IU/mL Penicillin/Streptomycin and 20% L929 supernatant. Media were replaced after 3 days. On day 6, differentiated macrophages were washed in phosphate-buffered saline (PBS; Gibco) and detached using a cell scraper. Cells were plated at $0.7x10^6$/mL in DMEM without serum for 1 hour to allow adhesion before culturing in DMEM with 10% FCS ± 200nM dexamethasone for 24 hours prior to experimentation, a widely established protocol to enhance efferocytosis[12,22].

Mouse alveolar macrophages (AMs) were obtained by lung lavage with 10mL PBS/0.5mM EDTA. AMs were centrifuged at 350g for 5 minutes then resuspended in IMDM and incubated at 150,000 cells per well in a 96 well plate. After 1 hour, culture media was replaced with IMDM supplemented with 10% FCS prior to overnight incubation.

***In vitro* phagocytosis assays**

The analysis of phagocytosis of fluorescently-labelled apoptotic cells was performed using a modified previously described method[12]. Macrophages were stained with CellTrace Far Red (Thermo Fisher Scientific) as per manufacturer's instructions prior to the addition of apoptotic cells. Human eosinophil constitutive apoptosis was induced by overnight culture, while apoptosis of mouse bmEos was induced by overnight culture with 1μM budesonide in the absence of IL-5. Apoptosis was examined by Annexin-V and propidium iodide staining by flow cytometry. Apoptotic eosinophils were labeled with pHrodo as per manufacturer's instructions then washed and resuspended at $4x10^6$/mL (human eosinophils) or $5x10^6$ (mouse bmEos) in IMDM and co-incubated with macrophages for 1 hour with 33nM Protein S (Pros1) with or without 1μM BMS777607 (Selleck Chemicals) as per figure legends. After co-incubation, macrophages were detached with 0.05% trypsin/0.53mM EDTA and phagocytosis assessed by flow cytometry (BD LSR Fortessa, BD Biosciences)[23,24].

Western Blotting

Western blotting was performed as described[25,26]. Briefly, BMDMs were lysed in 0.1% Nonidet P40 containing a protease inhibitor cocktail[26]. Lysates were separated on a 12% Tris-HEPES Precise gel (Thermo Fisher Scientific) and transferred electrophoretically onto polyvinylidene difluoride (PVDF) membranes (Merck Millipore). Membranes were blocked with 5% non-fat milk (Marvel) in Tris-buffered saline (TBS)/0.1% Tween-20 before incubation with primary antibodies directed against Mer (1:000; AF591, R&D Systems) and β-actin (1:50,000; A1978, Sigma). This was followed by horseradish-peroxidase-conjugated secondary antibodies (1:2500; Dako) and incubation with ECL prime (GE Healthcare). Blots were exposed to light-sensitive film (MOL7016, SLS) and processed through an X-ray developer (Ecomax Processor, Photo Imaging Systems Ltd.).

***In vivo* model of allergic airway inflammation**

Experiments were performed in accordance with the UK Home Office Animals (Scientific Procedures) Act 1996, following review by local ethics committee. Wild type control (WT;

C57BL/6, Charles River Laboratories) and Mer^{KD} mice[27] (C57BL/6 background) were bred and maintained in specific pathogen-free conditions. Genotypes were confirmed prior to experimental procedures, with 6-8 week old mice used for *in vitro* experiments and 8-16 week old female mice used for *in vivo* experiments. OVA-induced allergic airway inflammation was modelled as described[9]. Briefly, mice were sensitized by intraperitoneal (*i.p.*) alum-precipitated (Alum Imject, Pierce Biotechnology) ovalbumin (OVA, Sigma; 20μg of OVA and 50μL of alum per mouse) on days 1 and 10 and challenged on days 22, 23 and 24 by intratracheal (*i.t.*) OVA (50μg). Mice were culled and bronchoalveolar lavage fluid (BALF) and lung tissue were acquired and processed as described[19].

BALF cells and lung interstitial inflammatory cells were incubated with combinations of antibodies against CD45/CD11b/Ly6G/Siglec-F/F4/80, with flow cytometric analysis performed in the presence of Flow-Check Fluorosphere counting beads (Beckman Coulter) to allow quantification of cell numbers. The resolution interval (R_i; the time for eosinophil numbers to decline to half-maximal numbers) was calculated as previously described[28]. BALF cytokines and mucus (MUC5AC) were quantified by ELISA (R&D Systems or Caltag Medsystems) or by forward phase protein microarray and expressed as either relative to lavage fluid control (PBS) or as relative expression in the Mer^{KD} mice compared to wild type controls at day 7 post OVA challenge (as indicated in the figure legends).

Lungs from separate animals were fixed in 10% formalin (Sigma) prior to sectioning and staining with hematoxylin and eosin (H&E) or periodic acid-Schiff (PAS). Histological scoring of alveolar and interstitial inflammatory cell infiltration was quantified after analysis by two independent, blinded observers, with quantification of airway mucus production performed using the mucus-goblet index as described[9].

Airway responsiveness to aerosolized methacholine was assessed in anesthetised and mechanically ventilated WT and Mer^{KD} mice after sensitization and challenge with OVA (Buxco Research Systems, Wilmington, NC). Lung resistance was determined and expressed relative to baseline values in the absence of methacholine with nebulized phosphate-buffered saline given as vehicle. Airway resistance, in the absence of methacholine challenge, was measured in OVA-naïve WT and Mer^{KD} mice.

In separate experiments dexamethasone (2mg/kg) was administered *i.p.* on day 3 post-OVA to induce eosinophil apoptosis, prior to quantification of BALF apoptotic eosinophil number by Annexin-V binding (of $CD45^{+ve}/CD11b^{+ve}/Ly6G^{-ve}/Siglec\text{-}F^{+ve}$ cells).

***In vivo* phagocytosis experiments**

Following isolation from peripheral blood human granulocyte apoptosis was induced by overnight culture prior to labelling with CellTracker™ Green as per manufacturer's instructions. Apoptosis was confirmed by Annexin-V and propidium iodide staining by flow cytometry. A total of 100,000 labelled apoptotic human cells were administered *i.t.* to naïve WT and Mer^{KD} mice. Mice were culled after 0 and 3 hours and BALF retrieved for analysis of $CellTracker^{TM}$ Green positive alveolar macrophages ($CD45^{+ve}/CD11c^{+ve}/CD11b^{-ve}$ cells), uncleared apoptotic cells (mouse $CD45^{-ve}$/CellTrackerTM $Green^{+ve}$ cells), recruited mouse granulocytes ($CD45^{+ve}/Ly6G^{+ve}$ cells) and necrotic debris ($EpCam^{-ve}/CD11c^{-ve}/F4/80^{-ve}$ low SSC/FSC events).

Data analysis

Data were analysed using Graphpad Prism (v5) with flow cytometry data analysed using FlowJo software (Treestar). All data are expressed as mean ± SEM and analysed by Student's *t*-test or analysis of variance (ANOVA) as appropriate with significance accepted at $P < 0.05$.

Results

Mer deficiency delays the resolution of allergic airway inflammation

To investigate the role of apoptotic cell clearance in the resolution of allergic airway inflammation, wild type (WT) and Mer deficient (Mer^{KD}) mice were sensitized and challenged with OVA prior to acquisition of tissue on days 1, 3, 7 and 10 post-OVA challenge (Figure 1A). While both WT and Mer^{KD} mice had similar peak BALF eosinophil numbers at day 3 post-OVA (1.27±0.12 vs. 1.12±0.23 $x10^6$/mL), delayed resolution of eosinophilic inflammation was observed in the Mer^{KD} mice at day 7 (Figure 1B and 1C). This revealed a Δ change in BALF eosinophil numbers between day 3 and day 7 of $1.79x10^5$/day in the WT mice vs. $0.63x10^5$/day in the Mer^{KD} mice, with a resolution interval (R_i) that was prolonged by 2 days in the Mer^{KD} mice (6.5 days vs. 8.5 days). Interestingly, numbers of interstitial eosinophils were similar at day 7 (0.58±0.06 vs. 0.73±0.12 $x10^6$/mL; Figure 1D). Similarly, H&E stained lung sections demonstrated predominantly perivascular inflammation, with no significant differences between WT and Mer^{KD} mice (Figure 1E-G).

The functional consequences of the delayed inflammation resolution in the Mer^{KD} mice were explored by measuring airway responsiveness in anesthetized, mechanically ventilated mice in response to aerosolized methacholine. Mer^{KD} mice that had been sensitised and challenged with OVA had increased airway resistance (Figure 2A). Baseline airway resistance (in the absence of methacholine) was unaltered in naïve Mer^{KD} mice (Figure S1) confirming that the observed increase in airway resistance was not innate, but specific to the presence of allergic airway inflammation. Airway mucus production was similar in both WT and Mer^{KD} mice (Figure 2B-E), although BALF total protein was increased in Mer^{KD} mice (671.7±99.9 vs. 990.0±28.8 μg/mL, $P<0.05$), consistent with the increased inflammation observed (Figure 2F).

Enhanced cytokine production is not a major feature of allergic inflammation in Mer^{KD} mice

Given that signalling via TAM receptors, including Mer, acts to suppress pro-inflammatory cytokine production[29], we analysed BALF cytokines to examine whether raised levels of cytokines were present in the Mer^{KD} mice and contribute to excess inflammation. Alterations in BALF proteins and cytokines were investigated by analysis of 43 separate targets at four separate time points. This analysis revealed that the overall pattern of cytokine expression

was similar between Mer^{KD} and WT mice (Figure S2) with 8 cytokines upregulated >10% and 21 downregulated >10% in the Mer^{KD} mice at day 7 (Figure 2G). Despite increased relative expression of MCP5 (CCL12), RANTES (CCL5) and MCP-1 (CCL2) in the BALF of Mer^{KD} mice at day 7, these chemokines/cytokines were expressed at low absolute levels (data not shown). Overall, we interpret these data as suggesting that enhanced cytokine production was not the major mechanism behind the delayed resolution of allergic inflammation observed in the Mer^{KD} mice.

Mer inhibition or deficiency impairs phagocytosis of apoptotic eosinophils

We next investigated the relevance of Mer-mediated apoptotic eosinophil clearance to the observed delayed resolution of allergic airway inflammation in Mer^{KD} mice. Loss of Mer expression on macrophages from Mer^{KD} mice was confirmed by Western blotting of bone marrow-derived macrophages (BMDMs) and by flow cytometry of alveolar macrophages (AMs; Figure S3A-C). WT AMs expressed both Mer and Axl, with Axl expression unchanged on AMs from Mer^{KD} mice (Figure S3B&C). To assess macrophage capacity for efferocytosis, BMDMs (Figure 3A) and AMs (Figure 3B) from WT and Mer^{KD} mice were co-cultured with pHrodo-labelled apoptotic eosinophils (Figure S4). To ensure that Mer-dependent efferocytosis was not limited by bridging ligand availability, exogenous Mer ligand (Protein S; Pros1) was also added to these experiments. Although we observed a significant component of Mer-independent phagocytosis of apoptotic eosinophils, around 30% of AM phagocytosis was Mer-dependent (Figure 3A&B). Furthermore, AMs treated with BMS777607 (a c-Met inhibitor that inhibits Axl, Tyro3 and Mer) displayed substantial inhibition of phagocytosis of apoptotic eosinophils (Figure S3D&E), consistent with the expression of both Mer and Axl by AMs. Inhibition of BMDM efferocytosis by BMS777607 was less marked, consistent with low level expression of Axl by these cells (data not shown) and previous data demonstrating that WT and $Axl^{-/-}$ BMDMs have similar rates of efferocytosis[12].

Mer augments apoptotic cell clearance *in vivo* to dampen inflammation

To further investigate the role of Mer-mediated engulfment of apoptotic eosinophils *in vivo*, the glucocorticoid dexamethasone was administered to OVA-sensitised and challenged mice at the peak of inflammation (day 3 post-OVA) to induce eosinophil apoptosis[10] (Figure 3C). BALF was acquired at 8, 16 and 24 hours post dexamethasone administration and eosinophils analysed for evidence of cellular death by Annexin-V binding (Figure S5). This revealed a

time-dependent increase in the percentage of Annexin-V positive eosinophils seen in BALF of Mer^{KD} mice (4.8±0.5% vs. 9.2±0.6 at 16h, $P<0.05$) (Figure 3D&E) consistent with a compromised capacity for eosinophil clearance in the absence of Mer.

Furthermore, direct *i.t.* administration of labelled human granulocytes which had undergone cell death (predominantly apoptosis; Figure S4B) to naïve WT and Mer^{KD} mice with tissue acquisition 3 hours later revealed a significant increase in total BALF cells in the Mer^{KD} mice (Figure 4A). Total BALF cell numbers in the WT mice 3 hours after apoptotic cell administration were near identical to those of mice that had been administered PBS as a control, indicating successful clearance of the dead cells in the presence of intact Mer-mediated efferocytosis (Figure 4A). In contrast, increased total numbers of cells were observed in the Mer^{KD} mice suggesting that the absence of Mer-mediated efferocytosis resulted in either failed clearance of the administered apoptotic cells, or recruitment of inflammatory cells in response to the apoptotic cells, or both. Indeed, alveolar macrophages ($CD45^{+ve}/CD11c^{+ve}$ cells, Figure S6A) from Mer^{KD} mice were characterised by reduced phagocytosis of the administered apoptotic cells *in vivo* (Figures 4B&C). Minimal phagocytosis was observed in lung interstitial macrophages, which also express Mer, from either WT or Mer^{KD} mice. This is consistent with their limited anatomical ability to access the airway lumen[30] (data not shown).

In addition, an increased proportion of the administered apoptotic cells (mouse $CD45^{-ve}/CellTracker^{+ve}$) were recovered in BALF from Mer^{KD} mice (Figure 4D), highlighting the importance of Mer in mediating apoptotic cell clearance within the airway lumen. In parallel, an increased percentage of mouse neutrophils ($CD45^{+ve}/Ly6G^{+ve}$ cells) was observed in BALF from Mer^{KD} mice (Figure 4E). This increase in neutrophils was not observed in BALF recovered from Mer^{KD} mice immediately after administration of apoptotic cells (0 hours post *i.t.* AC), confirming that neutrophils were recruited in a time-dependent fashion specifically in the Mer^{KD} mice in response to apoptotic cells (Figure 4E). Lastly, a population of low SSC/FSC events that did not express markers of alveolar macrophage or epithelial cell origin ($EpCam^{-ve}/CD11c^{-ve}/F4/80^{-ve}$) was present in the BALF from Mer^{KD} mice at 3 hours post *i.t.* AC. This population was minimal in the WT mice (Figure 4F and Figure S6B&C) at 3 hours and minimal at 0 hours in the Mer^{KD} mice (data not shown). As uncleared apoptotic cells can undergo necrosis and release damage-associated molecular patterns leading to the recruitment of inflammatory cells[31], we hypothesised that the low SSC/FSC events were necrotic debris

from the instilled human granulocytes. Consistent with this, cytocentrifuge preparations of flow-sorted low FSC/SSC events revealed only cellular debris, in comparison to sorted $CD45^{+ve}/F4/80^{+ve}/CD11c^{+ve}$ events which clearly demarcated the AM population (Figure S6D&E). Overall, these data demonstrate that Mer augments apoptotic cell clearance *in vivo* to dampen inflammation in response to dying cells.

Discussion

Restoration of tissue homeostasis following tissue injury or infection requires termination of pro-inflammatory signalling and resolution of the inflammatory response. Control of the resolution process is achieved through a combination of local production of pro-resolution mediators and apoptosis of recruited inflammatory cells, together with their phagocytic removal[2,32]. It is widely accepted that disruption of the processes underlying the timely resolution of inflammation represents a significant contributory factor to the development of many inflammatory diseases. One corollary of the pivotal role of dysregulated resolution of inflammation in disease pathogenesis is that pharmacological modulation of the processes underlying inflammation resolution represent an attractive strategy to attenuate ongoing inflammation and accelerate the restoration of tissue homeostasis[33,34]. In support of this suggestion induction of apoptosis of either neutrophils or eosinophils in mouse models of sterile, infectious or allergic inflammation results in reduced inflammation in the airways and accelerated resolution of inflammation[9,19,35].

Induction of granulocyte apoptosis during inflammation would exert beneficial effects by directly reducing the overall tissue burden of granulocytes and by limiting the release of cellular contents that contribute to further tissue damage and the development of persistent inflammation. Moreover, there may be additional, indirect effects as a consequence of phagocyte uptake of apoptotic cells. Both “professional” and “non-professional” phagocytes, including airway epithelial cells, can mediate apoptotic cell clearance through multiple molecular pathways[4,36]. Such functional redundancy is thought to reflect the importance of effective apoptotic cell removal in both homeostatic and inflammatory processes. Efferocytosis promotes the resolution process by modulation of phagocyte production and release of anti-inflammatory lipids and cytokines together with suppression of pro-inflammatory cytokine release[37,38]. In particular, the receptor tyrosine kinases Axl and Mer mediate clearance of apoptotic cells and membranes by dendritic cells and macrophages. Axl and Mer exhibit segregation in terms of both expression and activity in a variety of tissue settings, suggesting that they may perform distinct, yet complementary physiological roles[12]. Expression of Axl is strongly induced by TLR-ligands and has been shown to play a major role in immunosuppression during inflammation. In contrast, Mer is upregulated by liver X receptor (LXR) ligands and glucocorticoids and is thought to function predominantly in tissue homeostasis. However, antibody-mediated inhibition of Mer exacerbates inflammation

following LPS challenge in the lung and augmentation of Mer activity exerts protective effects[16,39]. Together with evidence that resolution-phase macrophages express high levels of Mer, these data suggest that Mer represents an important contributor to the process by which inflammation normally resolves.

In this study, we have examined the role of apoptotic cell clearance in the resolution of inflammation associated with airway allergy. We report a number of novel findings that extend our understanding of the role of apoptotic cell clearance and Mer-mediated signalling in inflammation and tissue repair. First, we have demonstrated that the onset of inflammation in response to OVA challenge in the lung is similar in the absence of Mer, with equivalent numbers of eosinophils present in the BALF of WT and Mer^{KD} mice at day 3. However, at later time points (day 7) BALF eosinophils persist in the Mer^{KD} mice, together with increased BALF protein levels. The presence of ongoing inflammation in the absence of Mer demonstrate that Mer is an important contributor to the efficiency of resolving eosinophilic inflammation in the airways. Yet, the cellular inflammation in Mer^{KD} mice challenged with OVA returned towards baseline levels by day 10, suggesting that Mer-independent mechanisms ultimately allow clearance of recruited eosinophils in Mer^{KD} mice. The lack of an effect of loss of Mer upon lung histology and on the numbers of tissue eosinophils may be due to different mechanisms involved in eosinophil clearance in the airways and in the interstitial regions. One possibility is that eosinophils exhibit differential susceptibility to apoptosis in these distinct micro-environments, with airway eosinophils being more sensitive to undergoing pharmacological induction of apoptosis and subsequent phagocytic clearance than interstitial eosinophils[9].

Second, contrary to expectation, we did not observe highly elevated pro-inflammatory cytokine profiles in the Mer^{KD} mice at any of the time points examined during the course of the OVA-induced inflammatory response. Although Mer has been reported to suppress macrophage TNF production (for example following LPS-induced inflammation in the peritoneal cavity or in the lung[27]) it is possible that there may be stimulus-specific effects and that Mer does not act to counter-regulate a Th2-mediated inflammatory response. Comparison of expression levels at day 7 revealed that some potentially important chemokines, such as CCL12 and CCL5 (RANTES), that may act to recruit eosinophils were present at elevated levels in Mer^{KD} mice. However, these chemokines were present at relatively low levels in both WT and Mer^{KD} mice. Similarly, CCL11 and IL-5 that are

important for eosinophil recruitment and survival were expressed at roughly equivalent or lower levels in Mer^{KD} mice at 7 days. We suggest that these changes in chemokine/cytokine profiles in the Mer^{KD} mice are unlikely to account for the significant differences in eosinophil numbers observed. Whether Mer-driven resolution of allergic inflammation is associated with changes in the production of pro-resolving lipid mediators remains to be determined, but in a model of sterile peritonitis Mer deficiency was associated with reduced levels of lipoxinA4 and Resolvin D1[40].

Third, the delayed resolution of inflammation we observe in Mer^{KD} mice was accompanied by increased airway resistance, suggesting that the altered inflammatory response in the absence of Mer has consequences in terms of lung function. Since airway resistance was similar in naïve WT and Mer^{KD} animals, Mer is unlikely to represent a dominant factor in regulating airway function under homeostatic conditions. However, perturbation of lung homeostasis following injury or infection could highlight the role for Mer in regulation of responses to airway challenge. We did not find any significant changes in airway mucus production between WT and Mer^{KD} mice. It is possible that the differences in airway inflammation and resistance we have observed do not impact upon mucus production, or that mucus production is a less sensitive indicator of altered inflammation resolution.

To investigate the underlying mechanism of Mer in the process of inflammation resolution, we directly tested whether induction of high levels of apoptosis in eosinophils would reveal differences in the capacity for clearance of apoptotic cells in Mer^{KD} mice. In these experiments, we treated animals with dexamethasone at the peak of BALF eosinophil recruitment and tracked the extent of apoptosis present in BALF. We observed approximately twice as many Annexin-V positive apoptotic eosinophils in Mer^{KD} mice when compared with WT, consistent with a compromised capacity for eosinophil clearance in the absence of Mer. Glucocorticoids also act to increase Mer expression and function in macrophages which would further highlight the effect of Mer deficiency in this experimental model. Although our experiments did not specifically examine the possibility that some dexamethasone-treated eosinophils were progressing directly to necrosis *in vivo* without having first undergone apoptosis (primary necrosis), this represents an important area for future study. Eosinophils activated by inflammatory mediators can undergo primary necrosis more readily[41,42] and our subsequent experiments demonstrate that apoptotic cells lose their membrane integrity *in vivo* to become necrotic (secondary necrosis[43]), with this effect being marked in the absence of

Mer-dependent cell clearance. Our data demonstrates that approximately 25-30 percent of the total capacity for apoptotic cell clearance of bone marrow-derived macrophages or alveolar macrophages is Mer-dependent. Assuming that Mer mediates a similar proportion of macrophage capacity *in vivo*, the extent of apoptosis occurring during resolution of an inflammatory response may be sufficient to overwhelm the Mer-independent phagocytosis component leading to enhanced necrosis and amplification of inflammation.

In this manuscript, we have identified a role for apoptotic cell clearance by Mer in allergic airway inflammation, demonstrating a delay in resolution of inflammation in Mer^{KD} mice. Together, our data demonstrate that apoptotic cell clearance by phagocytes directly contributes to the resolution of allergic airway inflammation, suggesting augmentation of apoptotic cell clearance as a potential therapeutic strategy for treating allergic inflammation in humans.

Acknowledgements

We thank Greg Lemke at the Salk Institute for Biological Studies, La Jolla, CA 92037 for providing the Mer^{KD} mice. Flow cytometry was performed in the QMRI Flow Cytometry and Cell Sorting Facility and the MRC Centre for Regenerative Medicine Flow Cytometry Core, with help and support from Shonna Johnston, Will Ramsey, Mari Pattison and Fiona Rossi.

References

1. Rosenberg HF, Dyer KD, Foster PS. Eosinophils: changing perspectives in health and disease. Nat Rev Immunol. 2013;13:9–22.

2. Felton JM, Lucas CD, Rossi AG, Dransfield I. Eosinophils in the lung - modulating apoptosis and efferocytosis in airway inflammation. Front Immunol. 2014;5:302.

3. Lambrecht BN, Hammad H. The immunology of asthma. Nat Immunol. 2015;16:45–56.

4. Juncadella IJ, Kadl A, Sharma AK, Shim YM, Hochreiter-Hufford A, Borish L, et al. Apoptotic cell clearance by bronchial epithelial cells critically influences airway inflammation. Nature. 2012;493:547–51.

5. Persson C, Uller L. Theirs But to Die and Do: Primary Lysis of Eosinophils and Free Eosinophil Granules in Asthma. Am J Respir Crit Care Med. 2014;189:628–33.

6. Duncan CJA, Lawrie A, Blaylock MG, Douglas JG, Walsh GM. Reduced eosinophil apoptosis in induced sputum correlates with asthma severity. Eur Respir J. 2003;22:484–90.

7. Fernandez-Boyanapalli R, Goleva E, Kolakowski C, Min E, Day B, Leung DYM, et al. Obesity impairs apoptotic cell clearance in asthma. J Allergy Clin Immunol. 2013;131:1041–1047.e3.

8. Huynh M-LN, Malcolm KC, Kotaru C, Tilstra JA, Westcott JY, Fadok VA, et al. Defective Apoptotic Cell Phagocytosis Attenuates Prostaglandin E2 and 15-Hydroxyeicosatetraenoic Acid in Severe Asthma Alveolar Macrophages. Am J Respir Crit Care Med. 2005;172:972–9.

9. Lucas CD, Dorward DA, Sharma S, Rennie J, Felton JM, Alessandri AL, et al. Wogonin Induces Eosinophil Apoptosis and Attenuates Allergic Airway Inflammation. Am J Respir Crit Care Med. 2015;191:626–36.

10. Meagher LC, Cousin JM, Seckl JR, Haslett C. Opposing effects of glucocorticoids on the rate of apoptosis in neutrophilic and eosinophilic granulocytes. J Immunol. 1996;156:4422–8.

11. McColl A, Bournazos S, Franz S, Perretti M, Morgan BP, Haslett C, et al. Glucocorticoids Induce Protein S-Dependent Phagocytosis of Apoptotic Neutrophils by Human Macrophages. J Immunol. 2009;183:2167–75.

12. Zagórska A, Través PG, Lew ED, Dransfield I, Lemke G. Diversification of TAM receptor tyrosine kinase function. Nat Immunol. 2014;15:920–8.

13. Lu Q, Lemke G. Homeostatic Regulation of the Immune System by Receptor Tyrosine Kinases of the Tyro 3 Family. Science. 2001;293:306–11.

14. Lu Q, Gore M, Zhang Q, Camenisch T, Boast S, Casagranda F, et al. Tyro-3 family receptors are essential regulators of mammalian spermatogenesis. Nature. 1999;398:723–8.

15. D'Cruz PM, Yasumura D, Weir J, Matthes MT, Abderrahim H, LaVail MM, et al. Mutation of the receptor tyrosine kinase gene Mertk in the retinal dystrophic RCS rat. Hum Mol Genet. 2000;9:645–51.

16. Lee Y-J, Han J-Y, Byun J, Park H-J, Park E-M, Chong YH, et al. Inhibiting Mer receptor tyrosine kinase suppresses STAT1, SOCS1/3, and NF-κB activation and enhances inflammatory responses in lipopolysaccharide-induced acute lung injury. J Leukoc Biol. 2012;91:921–32.

17. Grabiec AM, Denny N, Doherty JA, Happonen KE, Hankinson J, Connolly E, et al. Diminished airway macrophage expression of the Axl receptor tyrosine kinase is associated with defective efferocytosis in asthma. J Allergy Clin Immunol. 2017;140:1144–1146.e4.

18. Thorp E, Vaisar T, Subramanian M, Mautner L, Blobel C, Tabas I. Shedding of the Mer Tyrosine Kinase Receptor Is Mediated by ADAM17 Protein through a Pathway Involving Reactive Oxygen Species, Protein Kinase Cδ, and p38 Mitogen-activated Protein Kinase (MAPK). J Biol Chem. 2011;286:33335–44.

19. Lucas CD, Dorward DA, Tait MA, Fox S, Marwick JA, Allen KC, et al. Downregulation of Mcl-1 has anti-inflammatory pro-resolution effects and enhances bacterial clearance from the lung. Mucosal Immunol. 2013;7:857–68.

20. Dyer KD, Moser JM, Czapiga M, Siegel SJ, Percopo CM, Rosenberg HF. Functionally competent eosinophils differentiated ex vivo in high purity from normal mouse bone marrow. J Immunol. 2008;181:4004–4009.

21. Dransfield I, Zagórska A, Lew ED, Michail K, Lemke G. Mer receptor tyrosine kinase mediates both tethering and phagocytosis of apoptotic cells. Cell Death Dis. 2015;6:e1646.

22. Liu Y, Cousin JM, Hughes J, Damme JV, Seckl JR, Haslett C, et al. Glucocorticoids Promote Nonphlogistic Phagocytosis of Apoptotic Leukocytes. J Immunol. 1999;162:3639–46.

23. Michlewska S, Dransfield I, Megson IL, Rossi AG. Macrophage phagocytosis of apoptotic neutrophils is critically regulated by the opposing actions of pro-inflammatory and anti-inflammatory agents: key role for TNF-α. FASEB J. 2009;23:844–54.

24. Dorward D, Sharma S, Alessandri A, Rossi A, Lucas C. Assays of Eosinophil Apoptosis and Phagocytic Uptake. In: Walsh GM, editor. Eosinophils [Internet]. Springer New York; 2014. p. 177–95. (Methods in Molecular Biology). Available from: http://dx.doi.org/10.1007/978-1-4939-1016-8_16

25. Dorward DA, Rossi AG, Dransfield I, Lucas CD. Assessment of Neutrophil Apoptosis. In: Neutrophil Methods and Protocols [Internet]. Humana Press, Totowa, NJ; 2014 [cited 2017 Nov 21]. p. 159–80. (Methods in Molecular Biology). Available from: https://link.springer.com/protocol/10.1007/978-1-62703-845-4_10

26. Lucas CD, Allen KC, Dorward DA, Hoodless LJ, Melrose LA, Marwick JA, et al. Flavones induce neutrophil apoptosis by down-regulation of Mcl-1 via a proteasomal-dependent pathway. FASEB J Off Publ Fed Am Soc Exp Biol. 2013;27:1084–94.

27. Camenisch TD, Koller BH, Earp2 HS, Matsushima GK. A Novel Receptor Tyrosine Kinase, Mer, Inhibits TNF-α Production and Lipopolysaccharide-Induced Endotoxic Shock. J Immunol. 1999;162:3498–503.

28. Chiang N, Fredman G, Bäckhed F, Oh SF, Vickery T, Schmidt BA, et al. Infection regulates pro-resolving mediators that lower antibiotic requirements. Nature. 2012;484:524–8.

29. Rothlin CV, Ghosh S, Zuniga EI, Oldstone MBA, Lemke G. TAM Receptors Are Pleiotropic Inhibitors of the Innate Immune Response. Cell. 2007;131:1124–36.

30. Gibbings SL, Thomas SM, Atif SM, McCubbrey AL, Desch AN, Danhorn T, et al. Three Unique Interstitial Macrophages in the Murine Lung at Steady State. Am J Respir Cell Mol Biol. 2017;57:66–76.

31. Dorward DA, Lucas CD, Doherty MK, Chapman GB, Scholefield EJ, Morris AC, et al. Novel role for endogenous mitochondrial formylated peptide-driven formyl peptide receptor 1 signalling in acute respiratory distress syndrome. Thorax. 2017;72:928–36.

32. Hoodless LJ, Lucas CD, Duffin R, Denvir MA, Haslett C, Tucker CS, et al. Genetic and pharmacological inhibition of CDK9 drives neutrophil apoptosis to resolve inflammation in zebrafish *in vivo*. Sci Rep. 2016;6:srep36980.

33. Poon IKH, Lucas CD, Rossi AG, Ravichandran KS. Apoptotic cell clearance: basic biology and therapeutic potential. Nat Rev Immunol. 2014;14:166–80.

34. Fullerton JN, Gilroy DW. Resolution of inflammation: a new therapeutic frontier. Nat Rev Drug Discov. 2016;15:nrd.2016.39.

35. Rossi AG, Sawatzky DA, Walker A, Ward C, Sheldrake TA, Riley NA, et al. Cyclin-dependent kinase inhibitors enhance the resolution of inflammation by promoting inflammatory cell apoptosis. Nat Med. 2006;12:1056–64.

36. Han CZ, Juncadella IJ, Kinchen JM, Buckley MW, Klibanov AL, Dryden K, et al. Macrophages redirect phagocytosis by non-professional phagocytes and influence inflammation. Nature. 2016;539:570–4.

37. Basil MC, Levy BD. Specialized pro-resolving mediators: endogenous regulators of infection and inflammation. Nat Rev Immunol. 2016;16:51–67.

38. Quiros M, Nishio H, Neumann PA, Siuda D, Brazil JC, Azcutia V, et al. Macrophage-derived IL-10 mediates mucosal repair by epithelial WISP-1 signaling. J Clin Invest. 2017;127:3510–20.

39. Choi J-Y, Park H-J, Lee Y-J, Byun J, Youn Y-S, Choi JH, et al. Upregulation of Mer receptor tyrosine kinase signaling attenuated lipopolysaccharide-induced lung inflammation. J Pharmacol Exp Ther. 2013;344:447–58.

40. Cai B, Thorp EB, Doran AC, Subramanian M, Sansbury BE, Lin C-S, et al. MerTK cleavage limits proresolving mediator biosynthesis and exacerbates tissue inflammation. Proc Natl Acad Sci. 2016;113:6526–31.

41. Kano G, Almanan M, Bochner BS, Zimmermann N. Mechanism of Siglec-8–mediated cell death in IL-5–activated eosinophils: Role for reactive oxygen species–enhanced MEK/ERK activation. J Allergy Clin Immunol. 2013;132:437–45.

42. Radonjic-Hoesli S, Wang X, de Graauw E, Stoeckle C, Styp-Rekowska B, Hlushchuk R, et al. Adhesion-induced eosinophil cytolysis requires the receptor-interacting protein kinase 3 (RIPK3)–mixed lineage kinase-like (MLKL) signaling pathway, which is counterregulated by autophagy. J Allergy Clin Immunol [Internet]. 2017 [cited 2017 Nov 28].

43. Vanden Berghe T, Grootjans S, Goossens V, Dondelinger Y, Krysko DV, Takahashi N, et al. Determination of apoptotic and necrotic cell death in vitro and in vivo. Methods. 2013;61:117–29.

Figure legends

Figure 1. Mer^{KD} mice have delayed resolution of allergic airway inflammation *in vivo.* (A) Schema of experimental protocol. (B) Bronchoalveolar lavage fluid (BALF) eosinophils in wild type (WT) and (C) Mer^{KD} mice at 1, 3, 7 and 10 days post-ovalbumin (OVA) with delta (Δ) change in eosinophil number between day 3 and day 7 shown (n=7-10). (D) Interstitial eosinophils at day 7 in WT and Mer^{KD} mice (n=6-8). (E-F) Representative lung sections stained with haematoxylin and eosin (H&E) at day 7 post-OVA from (E) WT and (F) Mer^{KD} mice (scale bar 20μm, x100 original magnification). (G) Quantification of H&E stained lung sections at 7 days post-OVA treatment (n=5). Data are expressed as mean ± SEM, analyzed by 1-way analysis of variance (ANOVA) with Newman-Keuls Multiple Comparison Test (B, C) or by Student's t-test (D, G), *p<0.05, **p<0.01, ***p<0.001.

Figure 2. Mer^{KD} mice have exacerbated allergic airway responses. (A) Airway responsiveness to aerosolized methacholine was assessed in anesthetized and mechanically ventilated mice at 7 days post-OVA with lung resistance expressed relative to WT baseline (after nebulization of PBS without methacholine; n=4-5). (B-C) Representative day 7 lung sections stained with periodic acid Schiff (PAS) from (B) WT and (C) Mer^{KD} mice (scale bar 20μm, x200 original magnification). (D) Quantification of mucus production at day 7 as assessed by the mucus-goblet index (MGI) on PAS stained lung tissue sections (n=5). (E) Bronchoalveolar lavage fluid (BALF) Mucin5AC (MUC5AC; a mucus glycoprotein) and (F) total protein content were measured at day 7 post-OVA (n=6-8). (G) Cytokine array showing day 7 cytokines, chemokines & proteins upregulated in the MerKD mice depicted in red, those downregulated depicted in blue. Data are expressed as mean ± SEM, analyzed by 2-way analysis of variance (ANOVA) (A) or by Student's t-test (D, E, F), *p<0.05, **p<0.01.

Figure 3. Mer deficiency impairs phagocytosis of apoptotic eosinophils. (A) Phagocytic capacity of mouse bone marrow-derived macrophages (mBMDMs) from wild type (WT) or Mer^{KD} mice was assessed after co-culture with apoptotic mouse bone marrow-derived eosinophils in the presence of Protein S (Pros1) (n=4-5). (B) Phagocytic capacity of mouse alveolar macrophages (mAMs) from WT or Mer^{KD} mice was assessed after co-culture with apoptotic human eosinophils in the presence of Pros1 (n=4-5). (C) Schema of *in vivo* experimental protocol. (D) Annexin-V binding of bronchoalveolar lavage fluid (BALF)

eosinophils from WT or Mer^{KD} OVA-treated mice at 8, 16 and 24 hours post-dexamethasone (Dex) treatment (n=4-7). (E) Representative flow cytometry plots (Annexin-V/Siglec-F) from WT and Mer^{KD} mice showing the presence of Annexin-V^{+ve} eosinophils ($CD45^{+ve}$/$CD11b^{+ve}$/$Ly6G^{-ve}$/ Siglec-F^{+ve}/Annexin-V^{+ve} cells) at 16 hours post-Dex. Data are expressed as mean ± SEM, analyzed by Student's t-test (A, B) or 2-way analysis of variance (ANOVA) with Bonferroni test (D), *p<0.05, ***p<0.001.

Figure 4. Mer^{KD} alveolar macrophages have an impaired engulfment capacity causing delayed apoptotic cell clearance *in vivo*. CellTracker™ Green fluorescently labeled apoptotic human cells (AC) or PBS control were administered intratracheally (*i.t.*) to naïve wild type (WT) and Mer^{KD} mice, with bronchoalveolar lavage fluid (BALF) collected after 3 hours. (A) Total BALF cell count (B) percentage of alveolar macrophages ($CD45^{+ve}$/$CD11c^{+ve}$/$CD11b^{-ve}$) phagocytosing labelled apoptotic cells and (C) representative flow cytometry plots (SSC/CellTracker™ Green) from WT-PBS, or *i.t.* AC treated WT and Mer^{KD} mice showing CellTracker Green positive (i.e. engulfing) alveolar macrophages. (n=3 WT-PBS; n=6-7 *i.t.* AC treated WT and Mer^{KD} mice). (D) Uncleared apoptotic cells ($CD45^{-ve}$/CellTracker™ $Green^{+ve}$ cells) in BALF after 3 hours (n=7-9). (E) Recruited mouse granulocytes ($CD45^{+ve}$/$Ly6G^{+ve}$ cells) in BALF (n=2-3). (F) Percentage of necrotic debris present in BALF 3 hours after apoptotic cell administration (n=8-10). Data are expressed as mean ± SEM, analyzed by Student's t-test, *p<0.05, ***p<0.001.

Supplementary Figure Legends:

Figure S1. Lack of Mer has no effect on airway resistance in naïve mice. Relative airway resistance was assessed in anesthetized and mechanically ventilated naïve WT and Mer^{KD} mice in the absence of methacholine and expressed relative to WT mice values. Data are expressed as mean ± SEM, analyzed by Student's t-test (n=6-7).

Figure S2. Time course of BALF cytokines and proteins in wild type and Mer^{KD} mice post-ovalbumin. BALF cytokines and proteins were measured by forward phase protein array (n≥3 per genotype at each timepoint) with red depicting high expression and green low expression.

Figure S3. Confirmation of Mer expression on bone marrow derived and alveolar macrophages from WT mice and absent expression in Mer^{KD} mice. (A) Western blot of lysates of BMDMs from wild type (WT) or Mer^{KD} mice. (B-C) Representative flow cytometry histograms of AMs isolated from WT and Mer^{KD} mice showing (B) Mer and (C) Axl expression. (D) Bone marrow-derived macrophages (mBMDMs) from WT or Mer^{KD} mice were co-cultured with apoptotic mouse bone marrow-derived eosinophils in the presence of Protein S (Pros1) with or without BMS777607 (BMS; c-met inhibitor to inhibit Axl/Tyro3/Mer) (n=4-5). (E) Alveolar macrophages (mAMs) from WT or Mer^{KD} mice were co-cultured with apoptotic human eosinophils in the presence of Pros1 with or without BMS777607 (n=4-5). Data are expressed as mean ± SEM, analyzed by 2-way analysis of variance (ANOVA) with Bonferroni test (E, F), **p<0.01, ***p<0.001.

Figure S4. Analysis of human granulocyte viability, apoptosis and necrosis. (A) Assessment of viable (Annexin-V^{-ve}/PI^{-ve}), apoptotic (Annexin-V^{+ve}/PI^{-ve}) and necrotic (PI^{+ve}) human eosinophils by Annexin-V/propidium iodide (PI) staining by flow cytometry prior to incubation with mouse alveolar macrophages and (B) human granulocytes undergoing constitutive apoptosis prior to intratracheal administration to mice. Example flow cytometry plots and cumulative data shown (n=2-4). (C) Representative cytocentrifuge preparation of aged human eosinophils, black arrow highlights an apoptotic eosinophil with typical cellular shrinkage and nuclear condensation (scale bar 20μm, x1000 original magnification).

Figure S5. Flow cytometry gating strategy to identify Annexin-V^{+ve} eosinophils. Representative flow cytometry plots showing the gating strategy used to identify Annexin-V^{+ve} eosinophils (CD45^{+ve}/CD11b^{+ve}/Siglec-F^{+ve}/Ly6G^{-ve}/Annexin-V^{+ve} cells) within BALF of OVA-challenged mice.

Figure S6. Identification of alveolar macrophage phagocytosis of apoptotic cells, uncleared human granulocytes and necrotic debris *in vivo*. (A) Representative flow cytometry plots showing the gating strategy used to identify alveolar macrophages (CD45^{+ve}/CD11c^{+ve}/CD11b^{-ve} cells) within the bronchoalveolar lavage fluid (BALF) of apoptotic cell-treated mice. (B-C) Representative flow cytometry plots demonstrating AM population (red) and low FSC/SSC necrotic debris population (blue) in BALF from (B) WT and (C) Mer^{KD} mice 3 hours after administration of apoptotic cells. (D-E) Cytocentrifuge

preparations of FACS sorted (D) low FSC/SSC events and (E) $CD45^{+ve}/F4/80^{+ve}/CD11c^{+ve}$ alveolar macrophages (scale bar 40μm, x1000 original magnification).

Figure 1

Felton JM *et al.*

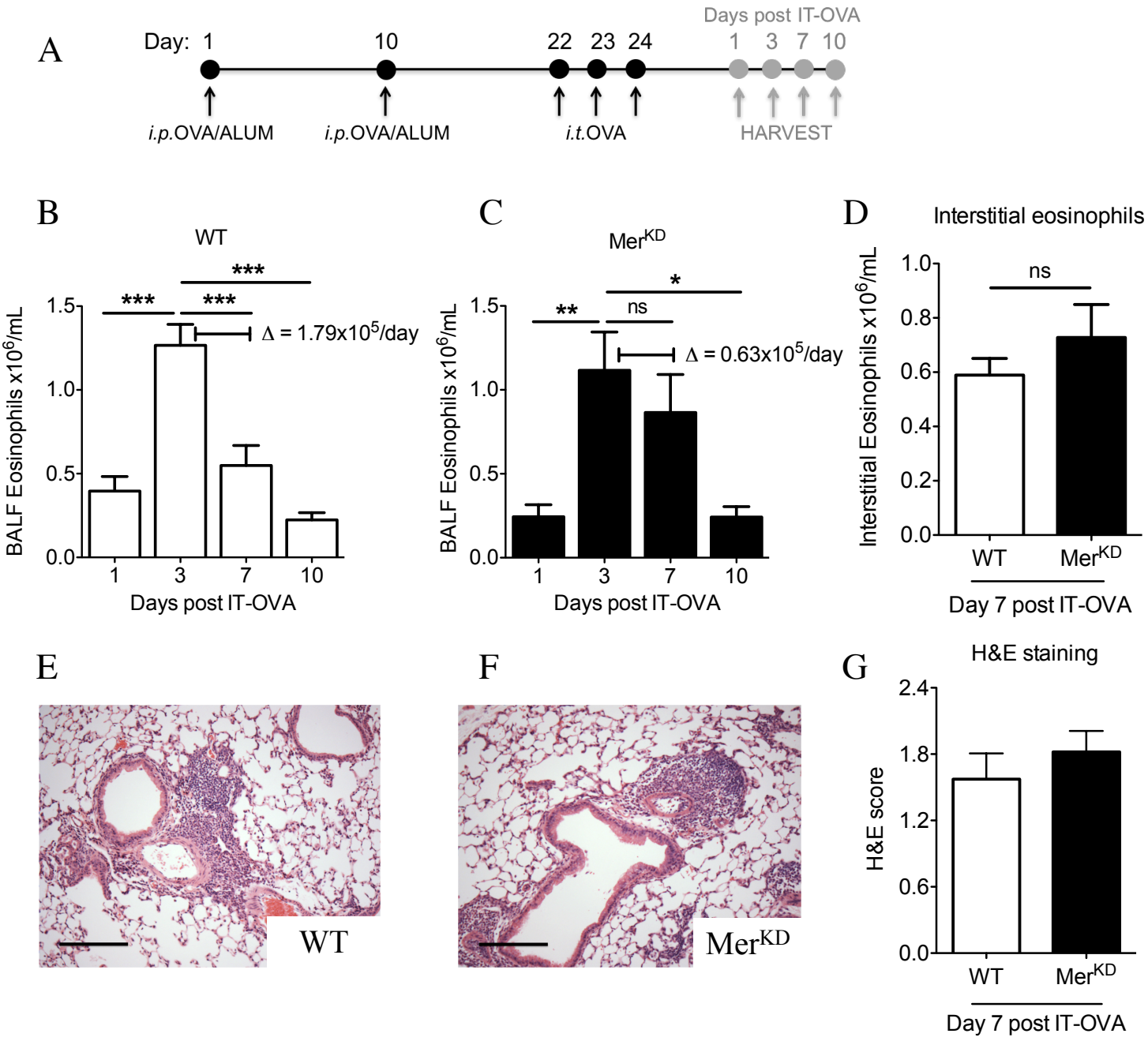
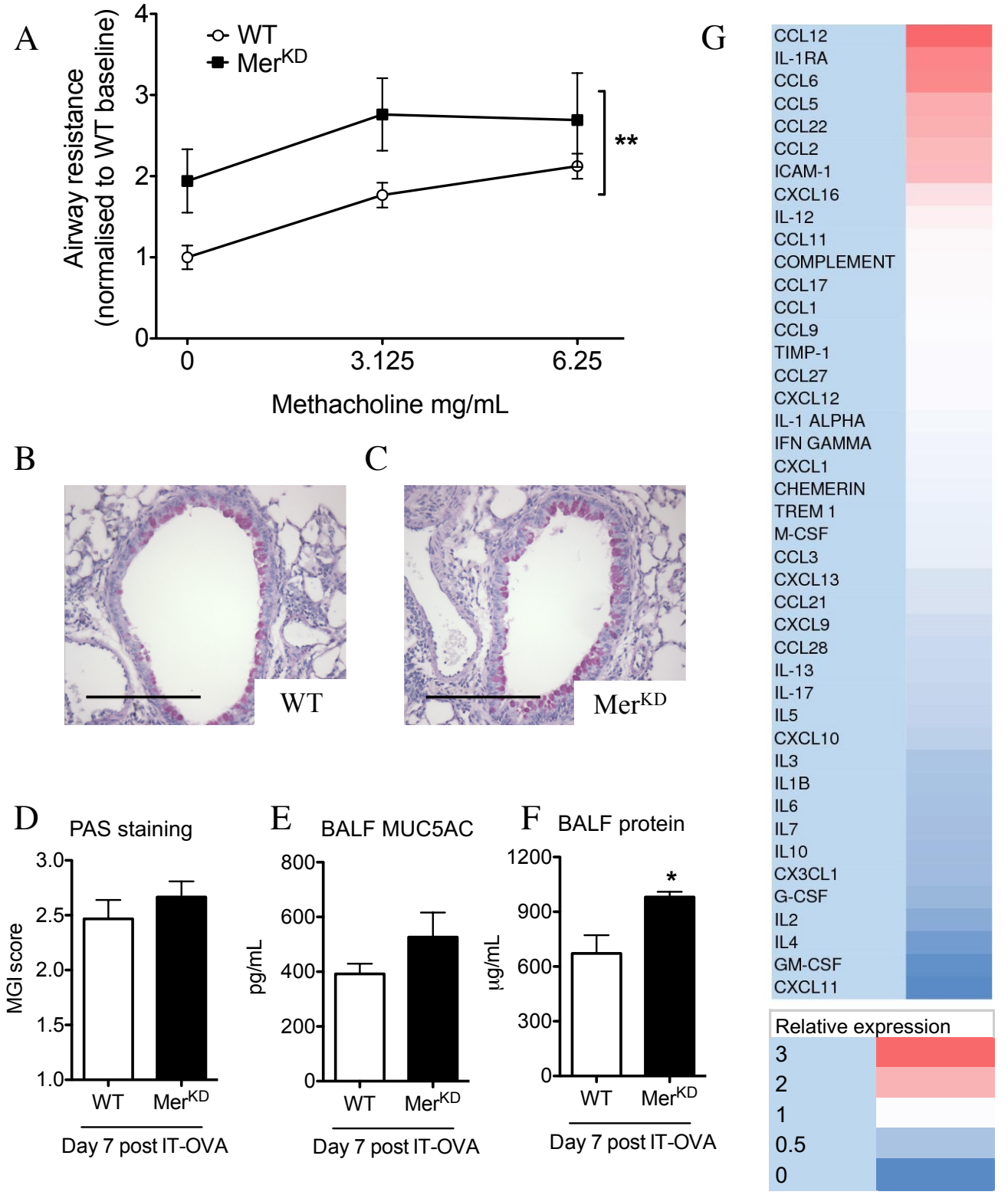


Figure 2 Felton JM *et al.*



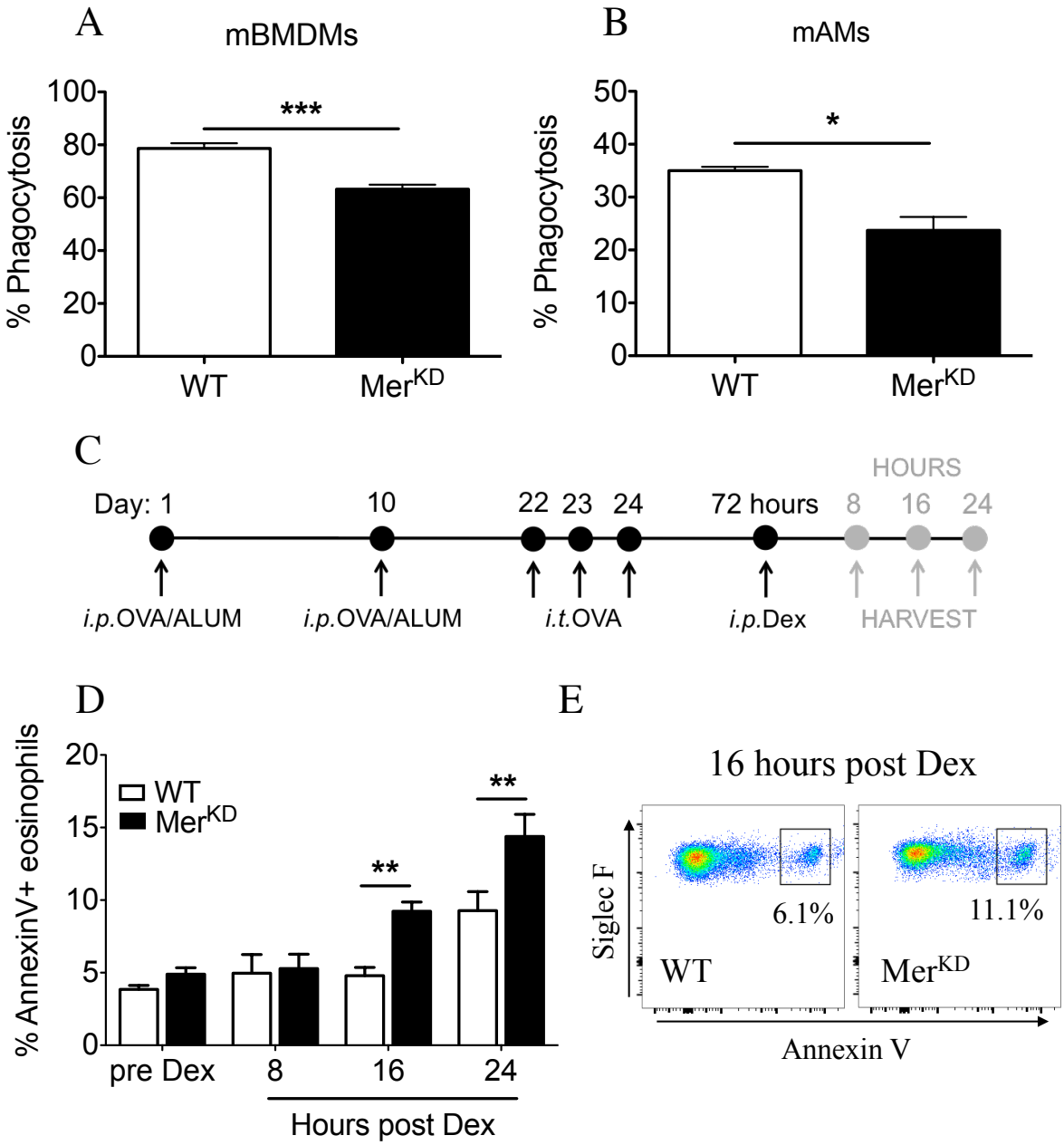
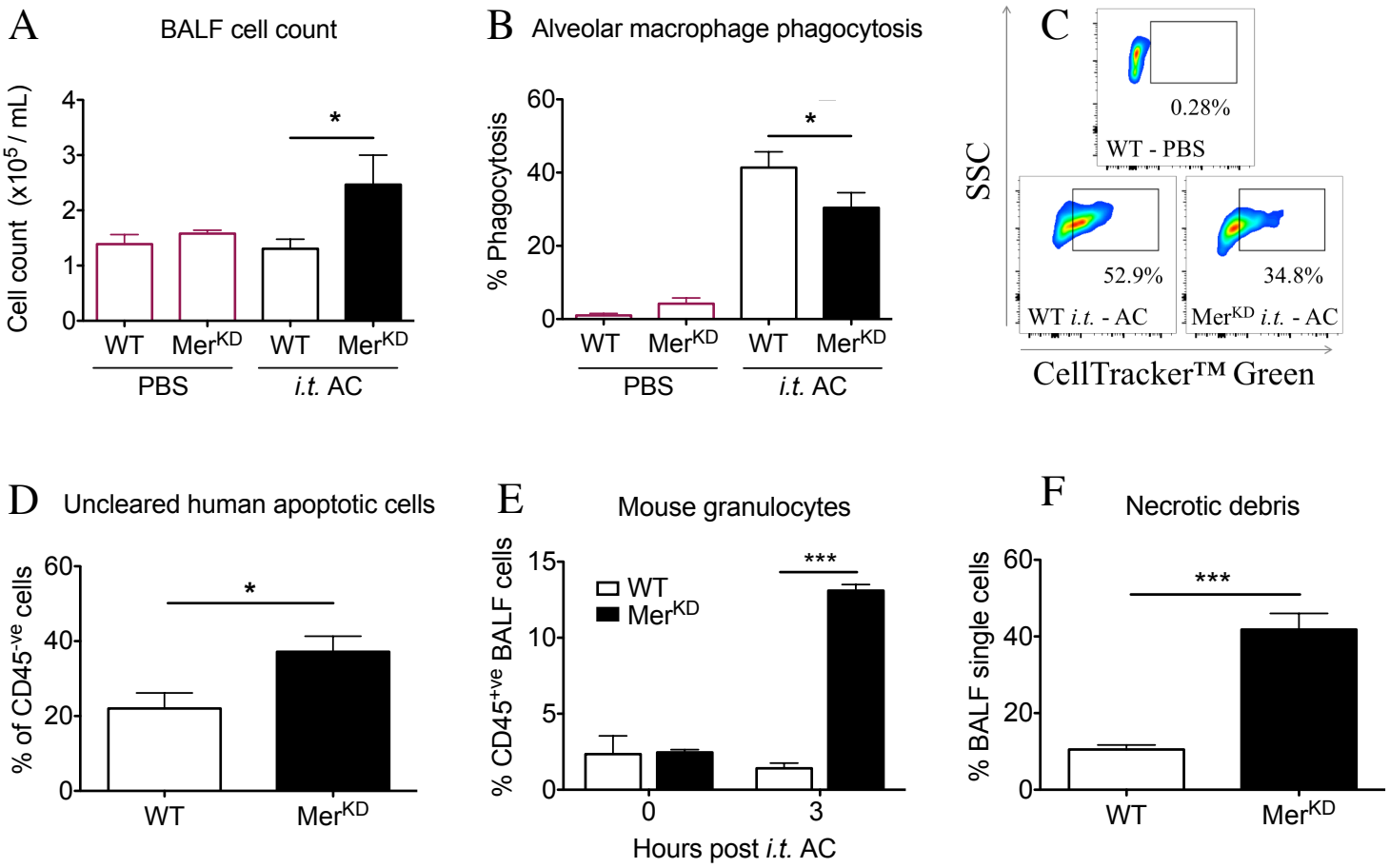
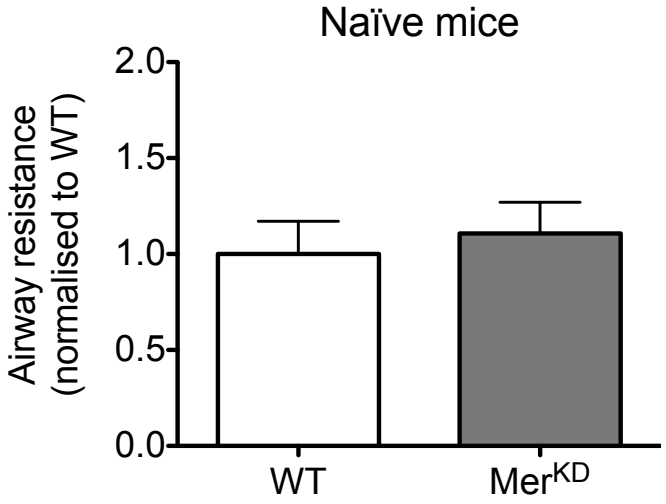

A
mBMDMs
% Phagocytosis
100
80
60
40
20
0
***
WT
Mer^KD
B
mAMs
% Phagocytosis
50
40
30
20
10
0
*
WT
Mer^KD
C
HOURS
Day: 1
10
22 23 24
72 hours
8 16 24
i.p.OVA/ALUM
i.p.OVA/ALUM
i.t.OVA
i.p.Dex
HARVEST
D
% AnnexinV+ eosinophils
20
15
10
5
0
WT
Mer^KD
**
**
pre Dex
8
16
24
Hours post Dex
E
16 hours post Dex
Siglec F
6.1%
11.1%
WT
Mer^KD
Annexin V

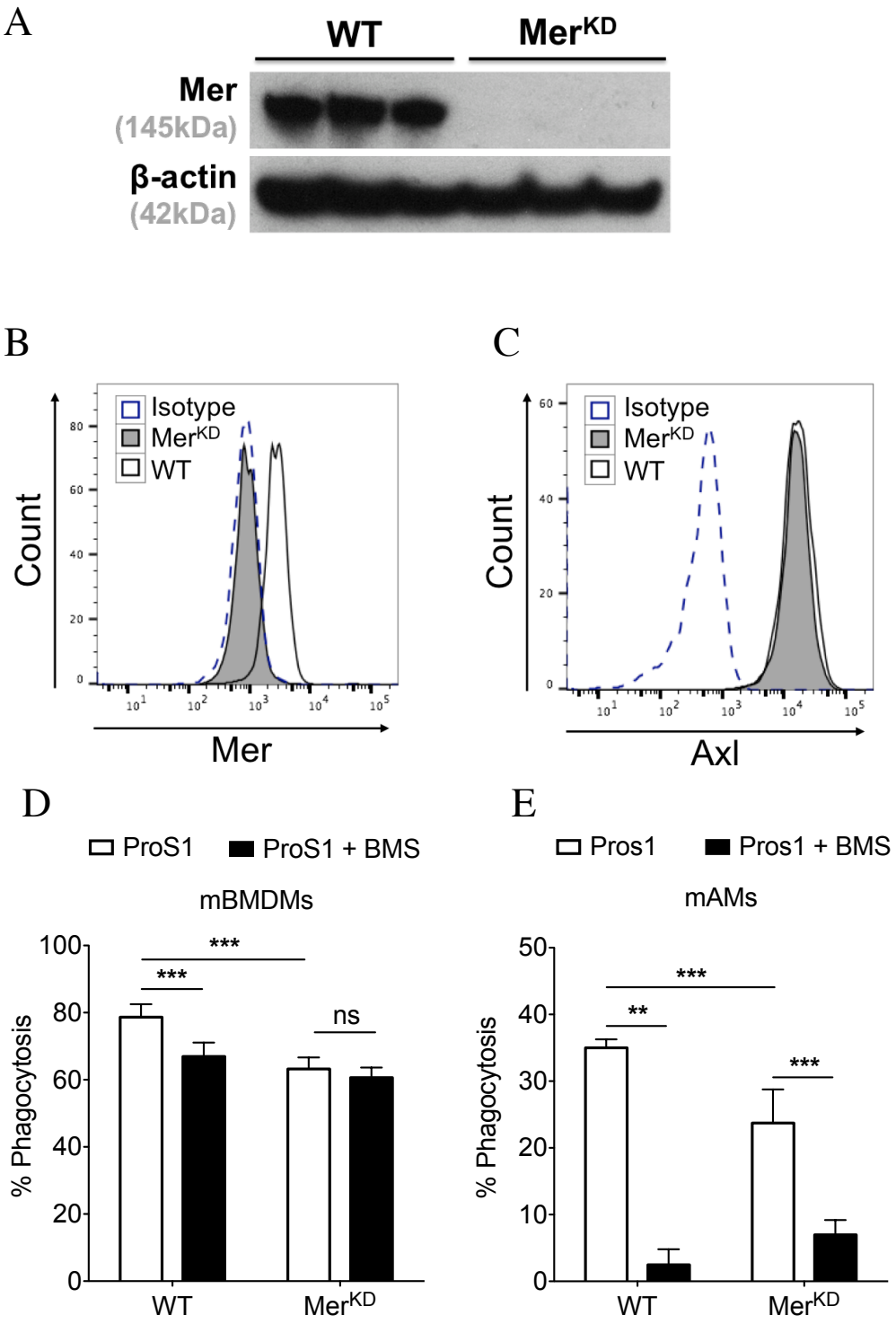
Figure 4 Felton JM *et al.*



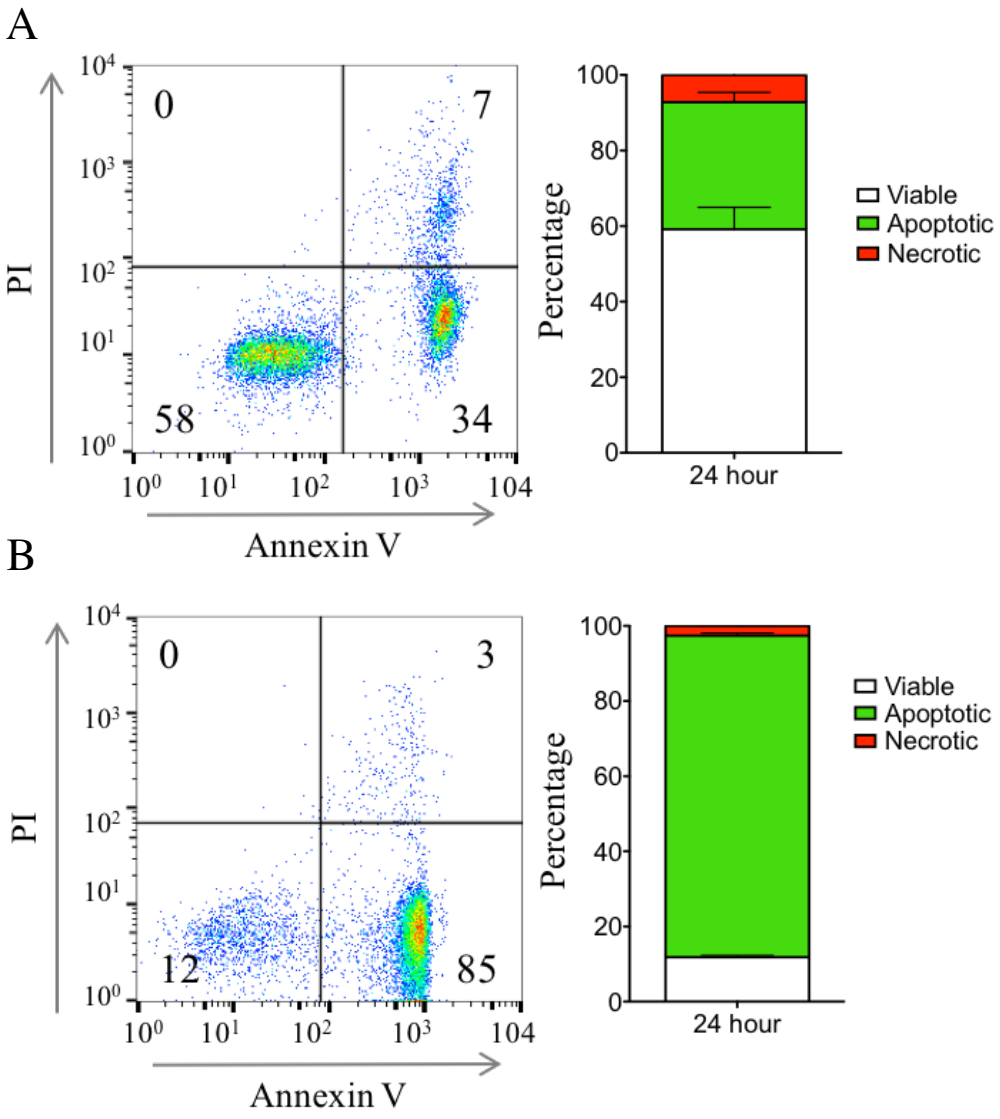
Supplementary Figure S1 Felton JM *et al.*



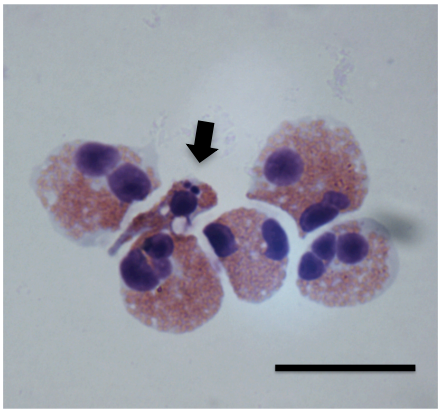
Supplementary Figure S2 Felton JM *et al.*


A
WT
MerKD
Mer
(145kDa)
β-actin
(42kDa)
B
Isotype
MerKD
WT
Count
Mer
C
Isotype
MerKD
WT
Count
Axl
D
ProS1
ProS1 + BMS
mBMDMs
% Phagocytosis
***
***
ns
WT
MerKD
E
Pros1
Pros1 + BMS
mAMs
% Phagocytosis
***
**
***
WT
MerKD

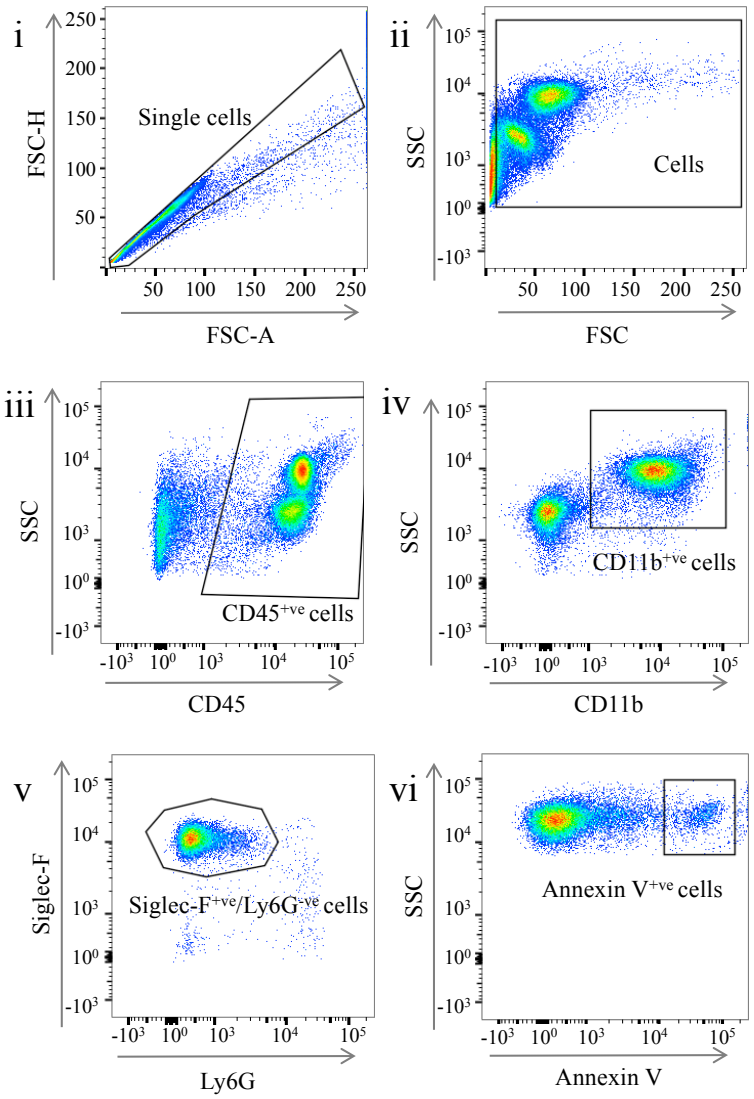
Supplementary Figure S4 Felton JM *et al.*



C



Supplementary Figure S5 Felton JM *et al.*



Supplementary Figure S6 Felton JM *et al.*